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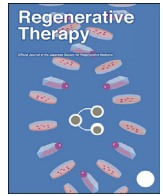
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Review

Biomaterial-based delivery systems of nucleic acid for regenerative research and regenerative therapy

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ABSTRACT

Regenerative medicine is a new and promising medical method aiming at treating patients with defective or dysfunctional tissues by maintaining or enhancing the biological activity of cells. The development of biomaterial-based technologies, such as cell scaffolds and carriers for drug delivery system, are highly required to promote the regenerative research and regenerative therapy. Nucleic acids are one of the most feasible factors to efficiently modify the biological activity of cells. The effective and stable delivery of nucleic acids into cells is highly required to succeed in the modification. Biomaterials-based non-viral carriers or biological carriers, like exosomes, play an important role in the efficient delivery of nucleic acids. This review introduces the examples of regenerative research and regenerative therapy based on the delivery of nucleic acids with biomaterials technologies and emphasizes their importance to accomplish regenerative medicine.

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Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated systems; DDS, drug delivery system; ECM, extracellular matrix; lncRNA, long non-coding RNA; miRNA, microRNA; mRNA, messenger RNA; MSC, mesenchymal stem cells; PEG, polyethylene glycol; PLGA, poly(d,l-lactic acid-co-glycolic acid); RISC, RNA-induced silencing complex; RNAi, RNA interference; siRNA, small interfering RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nucleases.

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1. Introduction

Regenerative medicine is to regenerate or repair the injured or lost tissues and substitute organ functions by maintain or augment the biological activity of cells in the site to be regenerated and repaired. It is highly expected that the regenerative medicine may resolve the problem of present advanced therapies, such as reconstruction therapy and organ transplantation.

There are two fields in regenerative medicine; regenerative research and regenerative therapy. The regenerative research scientifically supports the next generation of regenerative therapy and basically can be achieved through *in vitro* induction of cellular activity. Regenerative research composes of two objectives; the biological research of cells to make clear their activity for tissue regeneration and the drug discovery to efficiently evaluate drug action and toxicity with active cells. On the other hand, the objective of regenerative therapy is to treat diseases by the *in vivo* induction of cellular activity for tissue regeneration. In these contexts, it is important to develop technologies which enable to maintain or augment the biological activities of cells necessary for both the regenerative research and regenerative therapy.

The biological activity of cells is modulated by their interaction with bio-functional molecules and the surrounding microenvironment. For the successful modification of cellular biological activity, it is highly required to allow cells to effectively interact with bio-functional molecules and give good conditions for cells to augment the activities. Technologies based on biomaterials, defined as materials which are used contacting and interacting with the biological components, play an important role in the artificial modification of cellular biological activities for regenerative medicine.

In the case of modification of cellular biological activities by the bio-functional molecules, the biomaterials-based drug delivery system (DDS) technology can be utilized aiming at the effective delivery to the target cells. The objectives of DDS include the controlled release, the life-time extension, the accelerated permeation and absorption, and the targeting of drug. All of DDS objectives are applicable for the modification of cell biological activities based on the bio-functional molecules. The bio-functional molecules include low-molecular-weight substances, immune-stimulators, growth factors, antigens, agonists, antagonists, diagnostic agents, imaging probes, nucleic acids, and so on. The way how to interact with cells for the biological modification depends on the type of bio-functional molecules. For example, bio-signaling molecules of growth factors modify the biological activity of cells through the interaction with the receptors present on the surface of cells, followed by the induction of signaling pathways. On the other hand, several bio-functional molecules are required to be internalized into cells and interacted with intracellular substances to modify the biological activities of cells. Therefore, it is important to develop the DDS technology for the augmentation of cellular biological activities considering the manner of their interaction.

The biological activity of cells is also influenced by the surrounding microenvironment. It is well recognized that cells are present in the living tissue interacting with the extracellular matrix (ECM), which induces various types of cellular behaviors such proliferation, differentiation, and morphogenesis. Therefore, for the successful modification of cellular biological activities, it is indispensable to develop technologies to give cells a local microenvironment similar to the ECM (artificial scaffold) by making use of biomaterials. It has been demonstrated that the biological activity of cells is modified by

various characteristics of scaffolds, such as the charge, wettability, roughness, biological affinity of their surface or the stiffness, porosity, and pore size of the bulk property [1–3]. The cell scaffolds of biomaterials are used alone or by combining with the DDS technology.

In this review, nucleic acids of a bio-functional molecule to modify the biological activity of cells are focused. The strategies and examples of regenerative research and regenerative therapy based on the delivery of nucleic acids with biomaterials technologies are introduced to emphasize the importance of biomaterials-based DDS to achieve regenerative medicine.

2. Nucleic acids for the biological modification of cell activities

There are several types of nucleic acids which are involved in the biological modification of cellular biological activity through the regulation of cellular gene expression. The supplementation of gene or the corresponding messenger RNA (mRNA) into cells can increase the level of gene expression. On the other hand, the phenomenon of RNA sequence-specific suppression of gene expression level, so-called RNA interference (RNAi), has been discovered in mammalian cells [4]. RNAi has been recognized as a phenomenon that mRNA is sequence-specifically degraded by several small double strand RNAs to suppress the biological activity of the corresponding protein. Based on the understanding of RNA interfering, two main types of small RNAs such as small interfering RNAs (siRNA) and microRNAs (miRNA) have been developed to suppress the level of gene expression [5]. These RNAs form the RNA-induced silencing complex (RISC) with Argonaute and other proteins, followed by the binding to the target mRNA. The siRNA binds to the target mRNA with the full complementarity and cleaves at the 10–12 bases from the 5' end of binding site. The miRNA bind to the 3' untranslated region to inhibit the translation. Antisense oligonucleotides have two mechanisms to suppress gene expression [6]. One is the hybridization to inhibit the role of ribosome, alter the splicing, and inhibit the activity of ribonucleoprotein. The other is the degradation of mRNA by the enzymes of RNase H and RISC. The level of gene expression is also suppressed by a decoy of nucleic acid with the same sequence for the interaction with the transcription factor [7]. Taken together, various kinds of nucleic acids enable cell to modify their biological activity via the regulation of gene expression level.

It is well recognized that the nucleic acids in the naked form cannot effectively exhibit their biological activities in the living systems. A lot of technologies have been developed for the DDS modification of nucleic acids. The general strategies on the design of DDS for nucleic acids have been reviewed in various literature [8–11]. The DDS strategy of nucleic acids to achieve regenerative therapy and regenerative research is intensively introduced in the following sections.

3. Strategies of regenerative research with nucleic acid

The objective of regenerative research is to create cells or their constructs having high biological activities with biomaterials *in vitro* and further develop the drug discovery and the basic research of cell biology. Regenerative research with nucleic acids is being proceeded based on the fact that the fate of cells is regulated by nucleic acids which regulate the cellular gene expression (Fig. 1). The efficient delivery of nucleic acids will be able to create cells with interesting activities or elucidate molecular mechanisms underlying the biological behavior of cells. For example, nucleic acids reprogramming and differentiation technologies can genetically engineer to create a disease cell by cell isolated from a disease

patient [12]. Personalized drug discovery can be performed by using the disease cells created. In addition, recently, technologies to create a 3-dimensional (3D) tissue *in vitro* by combining cell with the 3D scaffold of biomaterial have been actively developed [13–15]. It has been demonstrated that the biological activities of 3D tissue-like cell constructs are high compared with those of original cells [16–19]. There are many approaches for the 3D construction of cells, which include cell aggregates, cell sheets, and cell microfabrication. Combination of DDS-modified nucleic acids with the 3D construct will further develop the research efficacy of drug discovery and cell biology. The imaging technology based on nucleic acids is also promising for the regenerative research. Modification with genes encoding fluorescent or luminescent proteins enables cells to demonstrate the distribution in the 3D construct, which is useful for the validation of 3D construct. When used in the drug discovery, the 3D construction of cells modified with a gene encoding a fusion protein of fluorescent protein and transcription factor, which is responsive to a biological phenomenon, enables the biological evaluation against drug candidates in the non-invasive manner.

4. Regenerative research based on nucleic acids of DDS

For the successful regenerative research with nucleic acids, it is indispensable to develop methods to efficient deliver the nucleic acid into cells of interest. Several biomaterials have been proposed for DDS of nucleic acids, which include cationized polymers [20–22], cationized liposomes [23–25], and ceramics [26]. However, since the biological expression of nucleic acids based on the carriers is transient, it is not always suitable to effectively carry out the regenerative research which is required to continue for long time period. As one trial, an intracellular controlled release of nucleic acid is considered to be promising.

We have prepared the nanoparticles of cationized gelatin hydrogel to succeed in the controlled release of nucleic acids inside cells [27]. The gene-carrying plasmid DNA incorporated in the nanoparticles was released in the cells with their degradation.

When internalized into bone marrow-derived mesenchymal stem cells, the cationized gelatin hydrogel nanoparticles were intracellularly degraded with time and the plasmid DNA incorporated was released in the controlled fashion. The time period of plasmid DNA release depended on the degradation extent of nanoparticles. In addition, this intracellular release technology with the cationized gelatin nanoparticles also extended the duration of gene suppression of siRNA [28].

In addition to the intracellular controlled release technology, we have applied a reverse transfection procedure established by Sabatini et al. [29] for the regenerative research. In the conventional procedure, transfection of nucleic acid is performed by adding nucleic acid complexed with a carrier to the culture medium of cells to be transfected. The reverse transfection is the transfection culture on the substrate immobilized nucleic acid complexed with a carrier. It has been revealed that the reverse transfection procedure has three advantages over the conventional transfection procedures [30]. First, the reverse transfection procedure maintained a high level of biological expression even in the presence of serum, where the level was decreased by the conventional method. Next, the biological expression of nucleic acid was extended by the reverse transfection method to a significantly great extent compared with that of the conventional method. Third, the cellular viability after the reverse transfection method was significantly higher than that by the conventional method. Considering the procedure of reverse transfection, this transfection method can be applied for the 3D culture substrate. We have succeeded in the reverse transfection in/on a non-woven fabric of polyethylene terephthalate [30], poly(D,L-lactic acid-co-glycolic acid) (PLGA) scaffold [31], and gelatin sponge [32]. It is no doubt that the technology of transfection of nucleic acids in the 3D scaffold will contribute the active promotion of regenerative research.

5. Strategies of regenerative therapy with nucleic acids

When the nucleic acid is applied for the regenerative therapy, the effective and stable delivery into the target cells *in vivo* is highly

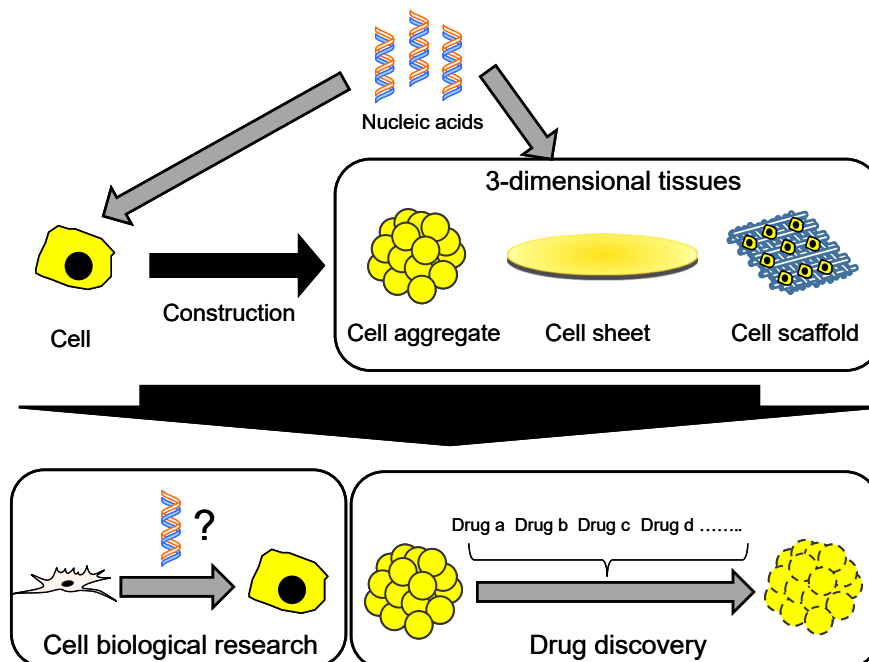


Fig. 1. The objective of regenerative research with nucleic acid is to effectively and stably deliver nucleic acids to cells or their 3D constructs *in vitro* by biomaterials-based DDS technologies. Cells or their 3D constructs modified with nucleic acids enable to promote the cell biological research and drug discovery.

desired. There are two approaches for the regenerative therapy with nucleic acid (Fig. 2). One is to directly deliver nucleic acids to cells present in the body. Types of cells to be delivered and nucleic acids to deliver depend on the strategy of regenerative therapy. To deliver the nucleic acids to tissue or stem cells which should be proliferated or differentiated for cell-based tissue regeneration, genes encoding the corresponding growth factors are used as a nucleic acid [33]. Recently, it has been demonstrated that one or several transcription factors or miRNA can control the fate of cells. Especially, a biological phenomenon, called as “direct reprogramming”, has been paid much attention. The direct reprogramming is the direct conversion from a terminally-differentiated cells to ones of different lineages. Many transcription factors and miRNA which enable cells to induce the direct reprogramming, have been discovered in various lineages [34]. The induction of “*in vivo*” direct reprogramming allows fibroblasts of a body cell which do not always contribute to the cell-based tissue regeneration.

As described in the Introduction section, the regulation of cellular microenvironment is crucial for cellular activity-based tissue regeneration. For example, angiogenesis plays an important role in tissue regeneration, because it enables cells to supply the oxygen and nutrient to the tissue as well as remove the wastes from the tissue. Many researches have been reported that the delivery of genes encoding angiogenic growth factors promotes the tissue regeneration [35]. In addition, recently much attention has been paid in the regulation of inflammatory environment governed by immune cells for the tissue regeneration [36]. Various types of immune cells, such as dendritic cells, macrophages, monocytes, neutrophils, and T cells etc., have been transfected with gene encoding factors affecting anti-inflammatory environments [37–39].

Cells transplantation is one of the most feasible approaches for the regeneration therapy since cells themselves have good therapeutic potentials in terms of their inherent targetability to the site injured or biological properties. However, the therapeutic efficacy of transplanted cells is not always as high as expected, which is one of the largest problems in cell therapy. This is because the survival ratio of transplanted cells is low, and consequently the biological activities of cells are not always achieved in the body as strongly as

those expected from the *in vitro* activities. There are two approaches to tackle the problem by making use of nucleic acids. One is to create an angiogenic or anti-inflammatory microenvironment by the transfection of nucleic acids to resident cells as described above. The other is to prepare cells biologically modified with nucleic acids before their transplantation. Genes encoding humoral factors to promote the tissue regeneration or reduce the suppression of cell biological activity associated with transplantation, have been selected for nucleic acids [40–42].

The exosome is one of the extracellular vesicles secreted from cells and includes various intracellular biomolecules, such as proteins, lipids, and nucleic acids including non-coding RNAs, such as miRNA and long non-codingRNAs (lncRNAs) [43,44]. The exosome is advantageous over other genetic substances in terms of the biological stability and specific information reflected to secreting cells. In addition, recently the intercellular communication by the exosome has been demonstrated [45–47]. In this context, the exosome can be expected to be a genetic substances used for gene-related therapeutic approaches [48]. Recently, trials on regenerative therapy have been performed by making use of exosomes obtained from somatic stem cells [49,50].

For successful cell therapy, it is indispensable to elucidate the therapeutic mechanism of cells transplanted by utilizing imaging technologies. Delivery of genes encoding fluorescent or luminescent proteins and transporter proteins for the uptake of radioactive substances has been performed to visualize and trace of cells without any invasiveness [51,52].

6. Regenerative therapy based on nucleic acids of DDS

Nanoparticle-based carriers, biodegradable polymer-based carriers, and biological carriers, such as cells and exosomes [53–58], have been mainly used to achieve regenerative therapy based on nucleic acids. In this section, material design of these carriers and concrete examples of regenerative therapy are introduced.

Since naked nucleic acids cannot always function in biological systems, the active usage of DDS is highly required for the nucleic acid-based regenerative therapy. There are two types of

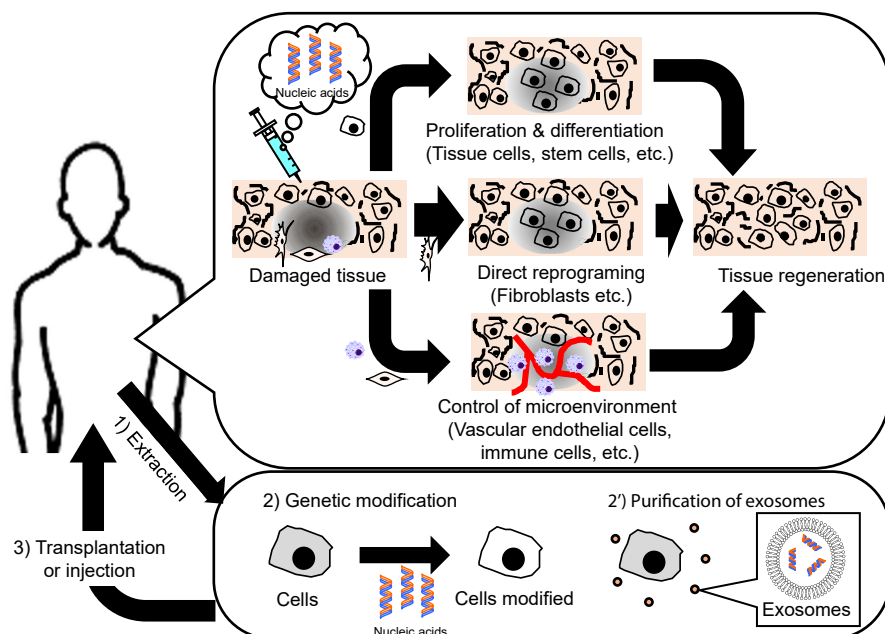


Fig. 2. There are two approaches for the regenerative therapy with nucleic acid; i) the direct delivery of nucleic acids to cells *in vivo* and ii) the transplantation of cells after extraction and modification with nucleic acids or the injection of exosomes from cells extracted.

administration procedures in regenerative medicine; local and systemic administrations. So far, few researches have been performed on the regenerative therapy based on the systemic administration of nucleic acids combined with DDS. In the case of local administration of nucleic acid, it is necessary to consider the technology how to avoid the diffusion away from the injection site and the degradation by nucleases. Complexation based on the electrostatic interaction or encapsulation of nucleic acids with biomaterial carriers has been explored as one trial to tackle these problems. The large size of complexes and the steric hindrance of biomaterial chains present on the surface of complexes can avoid the diffusion away from the injection site and the degradation by nucleases, respectively. For example, Uchida et al. have developed a nanomicelle carrier for mRNA [59]. The nanomicelle has an mRNA-containing inner core which is surrounded by the layer of polyethylene glycol (PEG). The PEG chain of nanomicelle makes mRNA stable even in the body. This nanomicelle system has been applied for the regenerative therapy in the brain as well as cartilage [60,61]. In addition, polymeric nanoparticles and liposomes have been used for the *in vivo* effective delivery of nucleic acid to achieve the tissue regeneration [33,62–64].

When injected into the body, a nucleic acid in the solution form rapidly diffused and disappeared from the injection site, resulting in the decreased biological activity. Considering the regenerative therapy based on the nucleic acid, the expression of biological activity induced by the nucleic acid in the target cells is highly required to maintain over several weeks or more. One of the promising ways is to make the nucleic acid to remain around the injection site and continuously act on the target cells. The controlled release technology of DDS enables a nucleic acid to maintain and release at the injection site in the controlled manner. This technology can reduce the adverse effects by the single bolus administration and the repeated administration. There are three types of carriers for controlled release; non-biodegradable polymers, biodegradable polymers, and ceramics. There are several studies on controlled release of drug with non-biodegradable materials [65,66]. However, they are required to exclude after the complete release of incorporated drug. On the other hand, various biodegradable polymers have been used as carriers for the controlled release of nucleic acids, such as poly(lactic acid) [67], polyanhydride [68], poly(orthoester) [69], PLGA [70–80], poly(D,L-lactide-co-4-hydroxy-L-proline) [81], poly(1,8-octanediol-co-citrate) [82], oligo(poly(ethylene glycol) fumarate) [83], poly(2-aminoethyl propylene phosphate) [84], polypseudorotaxane [85], polysaccharide [86], silk elastin-like polymer [87], and atelocollagen [88–90]. It has been reported that some ceramics, such as calcium phosphate and calcium carbonates, are used for the controlled release of nucleic acid [91,92]. By using the release system of nucleic acids with the biodegradable polymers, the regenerative therapy for various tissues has been tried, such as the bone formation [80,93], the recovery of spinal cord [78], and angiogenesis [86,94].

We have developed a cationized gelatin hydrogel as a carrier for the controlled release of nucleic acid. Cationization of gelatin was performed to electrostatically interact with the negatively-charged nucleic acids. In the same fashion as other gelatin hydrogels previously reported [95–97], the *in vivo* degradation of gelatin hydrogels can be controlled by their crosslinking extents, while the time profile of nucleic acid release was well corresponded to that of hydrogel degradation [98]. Different from other controlled release systems, nucleic acids are released from the hydrogels in the state of complex with the cationized gelatin fragments, which prevents the enzymatic degradation by nucleases. Nucleic acids complexed with the cationized gelatin fragments can be easily internalized into cells by the electrostatic

interaction with the negatively charged cell surface. As a result, the augmented and extended biological expression of nucleic acid was achieved by the cationized gelatin hydrogel. We have confirmed that the profile of the hydrogel degradation and release of nucleic acid is not influenced by the shape and size of hydrogel [99]. So far, the controlled release system of nucleic acids with the cationized gelatin hydrogels has succeeded in the regeneration therapies for fibrosis [73,100–103], aortic aneurysms [104], and autoimmune alopecia [105].

The genetic engineering of somatic stem cells is also one of the promising approaches for regenerative therapy. Various methods to genetically engineer somatic stem cells have been reported [40–42]. Mesenchymal stem cells (MSC) of somatic stem cell, is a promising cell source and has been used for the regeneration of various tissues [106]. We have designed and prepared cationized pullulan as a non-viral carrier of nucleic acid for MSC [107]. The complexation with the cationized pullulan enabled a plasmid DNA to augment the expression level of MSC to a significantly high extent compared with that of LipofectAmine 2000® commercially available. The genetic engineering of MSC by cationized pullulan has demonstrated the efficient therapeutic effect for myocardial infarction [108], brain diseases [109], liver fibrosis [110], cartilage regeneration [111], and wound repair [112,113].

7. Closing remarks and future perspectives

In this review, materials, technologies, and methodologies for regenerative research and regenerative therapy by utilizing nucleic acids were summarized. There have been reported on several materials and methodologies based on nucleic acids, which will be useful and utilized in the future for regenerative research and regenerative therapy.

Genome editing is a methodology to achieve the deletion and insertion of specific sequence in the genome through the repair mechanisms (non-homologous end joining, homologous recombination, etc.) after DNA double strand break by the artificial restriction enzymes [114]. The restriction enzymes include zinc-finger nuclease (ZFN) [115], transcription activator-like effector nuclease (TALEN) [116], and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems (Cas) (CRISPR/Cas) [117]. It is no doubt that the genome editing methods will strongly contribute to the regenerative research.

Optogenetics are methodologies to control the biological activities of cells based on a light by the cellular expression of gene encoding the light-responsive protein. It has been demonstrated that the control of ion channel, second messenger, actin polymerization, and gene expression was achieved by this method [118]. The spatial control of biological activity for cells by light is advantageous over other methods. By utilizing this method, the preparation of 3D construct for the regenerative research, where biological activities of cells are spatially regulated by light, might be achieved.

With the progress of biology and chemistry on nucleic acid, various functional nucleic acids, which can regulate or detect the biological activities of cells through the specific interaction with target endogenous nucleic acids (mRNA or miRNA), have been easily designed and prepared. For example, Saito et al. have developed a technology to regulate or detect the biological activities by artificially creating functional RNA or its complex of protein [119,120]. The technology to estimate the sequence and structure of nucleic acid necessary to interact with target nucleic acids has been already established. By this advantage, the functional nucleic acids to any target nucleic acid can be easily created. The versatile functional nucleic acids will strongly support the regenerative research.

In future, the technological fusion of genome editing, optogenetics, and functional nucleic acids with DDS will achieve a new strategy of regenerative medicine. It is no doubt that the DDS for nucleic acids is crucial to promote regenerative research and regenerative therapy. However, the DDS for nucleic acids is not always perfect and has some rooms to be improved technologically and methodologically. In fact, most of regenerative research and regenerative therapy with nucleic acids have been carried out by using viral carriers so far. For the promotion of regeneration research and regeneration therapy to the clinical level, it would be necessary to actively utilize the carriers of biomaterials which are being used in clinics and derived from the body component, such as extracellular vesicles [53–58], may become required. In addition, it is important for several researchers with scientific backgrounds of materials, chemicals, physics, biology, and informatics to develop a new research field of regenerative medicine by efficient and substantial fusion to each other based on their own specialty.

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